Chapter 13

High-Throughput Screening of Substrate Specificity for Protein Tyrosine Phosphatases (PTPs) on Phosphopeptide Microarrays

Liqian Gao, Su Seong Lee, Jun Chen, Hongyan Sun, Yuliang Zhao, Zhifang Chai, and Yi Hu

Abstract

Phosphatases are a family of enzymes responsible for the dephosphorylation of biomolecules. Phosphatases play essential roles in cell cycle regulation, signal transduction, and cellular communication. In recent years, one type of phosphatases, protein tyrosine phosphatases (PTPs), emerges as important therapeutic targets for complex and devastating diseases. Nevertheless, the physiological roles, substrate specificity, and downstream targets for PTPs remain largely unknown. To demonstrate how microarrays can be applied to characterizing PTPs, we describe here a phosphopeptide microarray strategy for activity-based high-throughput screening of PTPs substrate specificity. This is followed by a kinetic microarray assay and microplate assay to determine the rate constants of dephosphorylation by PTPs. This microarray strategy has been successfully applied to identifying several potent and selective substrates against different PTPs. These substrates could be used to design potent and selective PTPs inhibitors in the future.

Key words High-throughput screening, Protein tyrosine phosphatases, Phosphopeptide microarrays, Substrate specificity

1 Introduction

Reversible phosphorylation of tyrosine residues in proteins plays essential roles in modulating cellular events including cell growth, cell differentiation, cell-cycle regulation, and immune response [1, 2]. Tyrosine phosphorylation by kinases accounts for less than 1 % of all cellular phosphorylation events in normal tissues, and can be significantly increased upon oncogenic transformation or growth factor stimulation [3–5]. On the other hand, dephosphorylation is a key process involved in cell signaling via removal of a phosphate group from a protein or other small molecules by a phosphatase. Two opposing enzyme superfamilies, protein tyrosine kinase (PTK) family and protein tyrosine phosphatase (PTP) family,

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control the physiological balance of tyrosine phosphorylation inside the cells [5]. It has been reported that both genetic mutation and overexpression of PTPs could be implicated in human diseases such as diabetes and cancer [6]. Therefore, PTPs have been recognized as important therapeutic targets.

Since different PTPs are highly homologous in their active sites, they present a daunting challenge to develop selective and potent small molecule PTP inhibitors. The successful development of PTP inhibitors relies on detailed understanding of the molecular mechanism by which a given PTP carries out its enzymatic reaction, as well as on the substrate specificity of PTPs. Compared with many well-studied kinases [7], phosphatases have not been extensively characterized and their cellular partners/substrate specificities remain to be further explored [6, 8–10]. Conventional strategies for determining substrate specificity of PTPs, such as combinatorial peptide libraries [11], phage display [12], and SPOT synthesis (a technique for parallel synthesis of peptide libraries on the membrane) [13], have some limitations in terms of the throughput and sensitivity of the assays. In recent years, Waldmann group and Yao group have independently developed peptide microarrays for large-scale profiling of phosphatase substrate specificities [1, 4, 8]. With these high-throughput screening approaches, the enzymatic activity of different classes of protein phosphatases, including PTPs and Ser/Thr phosphatases, could be thoroughly examined, both qualitatively and quantitatively, against a library of phosphopeptides.

The microarray is a well-established high-throughput screening platform with a diverse spectrum of biological applications across different biomolecule types [14]. Microarrays could comprise hundreds or even thousands of molecules that are immobilized as micrometer-sized spots on a planar surface. This compact format enables simultaneous processing of samples on a large scale and is therefore amenable to high-throughput screening [15–17]. More specifically, peptide microarray offers a unique and versatile platform for determining the peptide substrate fingerprints of enzymes. In this study, we have successfully extended the previous phosphopeptide microarray approach for an activity-based high-throughput study of the substrate specificity of PTPs. In Fig. 1, the biotin-containing peptides were synthesized, immobilized onto avidin-coated arrays and screened against PTPs of interest. There were 144 peptides extrapolated from the putative PTP protein substrates. Each PTP will dephosphorylate its own peptide substrate. After specific fluorescent staining of the phosphate group in the phosphopeptide, microarray scanning, and data analysis (Fig. 2), the preferred peptide substrates for each PTP could be readily identified. One of the most classical PTPs, T-cell protein

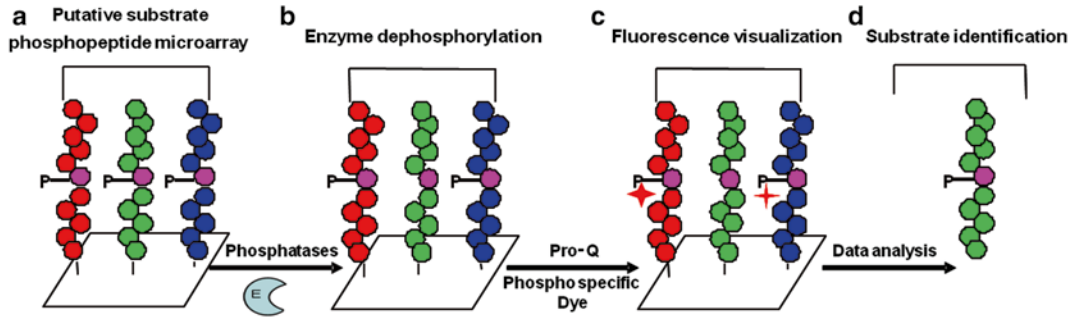

Fig. 1 The workflow of screening strategy: (**a**) Prepare a phosphopeptide microarray (P stands for phosphate). (**b**) Perform dephosphorylation assay. When incubated with PTPs, the immobilized phosphopeptides will be dephosphorylated based on PTP substrate specificity. (**c**) Perform Pro-Q dye assay, followed by fluorescence scanning with a microarray scanner to identify the phosphopeptides that are dephosphorylated by PTPs. (**d**) Carry out data analysis to identify the preferred peptide substrates for PTPs

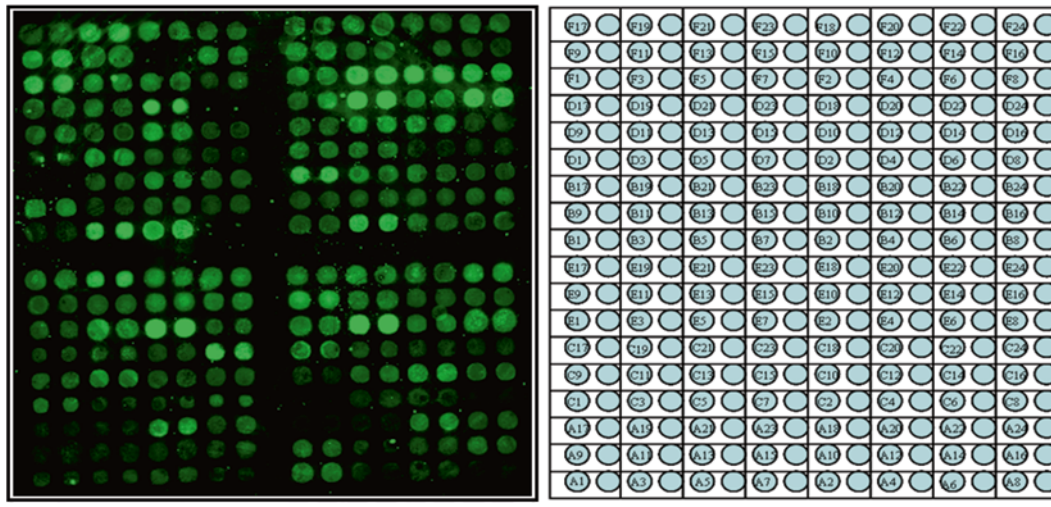

Fig. 2 Single-time-point (30 min) fingerprint results of the phosphopeptide microarrays treated with TCPTP (reproduced in part from ref. [4] with permission from John Wiley & Sons Inc.)

tyrosine phosphatase (TCPTP) was chosen in this study. Compared with the well-studied protein tyrosine phosphatase-1B (PTP1B), TCPTP is highly homologous to PTP1B and they share more than 70 % identity in protein sequence within the catalytic domain [18]. Through the example of TCPTP (Fig. 2), we will showcase the advantages of this phosphopeptide microarray approach in: (1) generating unique peptide substrate fingerprints of PTPs; (2) carrying out kinetic measurements and obtaining the kinetic constants of multiple peptides against PTPs; (3) identifying potent peptide substrates for PTPs.

2 Materials

2.1 Expression of TCPTP

1. Competent *E. coli* BL21 (DE3).
2. Construct of *TCPTP*.
3. Luria–Bertani (LB) media.
4. Kanamycin (antibiotic).
5. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
6. Circular shaker (Heidolph).

2.2 Purification of TCPTP

1. Chromatographic column (1.5 cm diameter × 10 cm length).
2. Nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Cat. No. 1018244).
3. His-tag purification lysis buffer (50 mM Tris base, 300 mM NaCl, pH 8.0).
4. His-tag purification wash buffer (50 mM Tris base, 300 mM NaCl, 20 mM imidazole, pH 8.0).
5. His-tag purification elution buffer (50 mM Tris, 300 mM NaCl, 200 mM imidazole, pH 8.0).
6. SDS gel (10–12 %).
7. Protein marker.

2.3 Preparation of Avidin Slides

1. Piranha solution (H_2SO_4:H_2O_2 = 7:3).
2. APTES solution (400 mL): 12 mL aminopropyltriethoxisilane dissolved in 380 mL 100 % ethanol and 8 mL H_2O.
3. Avidin solution (for 30 slides): 1.5 mg avidin, 1.47 mL of Milli-Q water, 30 μL of 0.5 M $NaHCO_3$ (pH 9.0).
4. Carboxylic acid activation solution: 400 mL of DMF (HPLC grade), 7.4 g succinic anhydride, and 18 mL of 1 M $Na_2B_4O_7$ (pH 9.0).
5. NHS activation solution: 15 mL of DMF, 565 mg O-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 173 mg *N*-hydroxysuccinimide (HOSu), and 550 μL of *N*,*N*-diisopropylethylamine (DIEA). This will give 100 mM of HBTU, 100 mM of HOSu, and 200 mM of DIEA in 15 mL of DMF.
6. Quenching solution: 2 mM aspartic acid in 0.5 M $NaHCO_3$ (pH 9.0).
7. 150 °C oven.
8. Slide dish.
9. Metallic tray.
10. Magnetic stirrer.

2.4 Microarray Spotting

1. Phosphopeptide library stock solutions.
2. Phosphate buffered saline (PBS) (pH 7.4).
3. 384-well polypropylene microarray plate.
4. Stealth Micro Spotting pins (Telechem International, cat. ID. SMP7B).
5. Arrayer (ESI SMA™, Ontario, Canada).

2.5 Pro-Q™ Assay

1. Pro-Q™ Diamond dye (Invitrogen, cat. No. P33300). This dye binds to phosphate on the phosphopeptide to produce a baseline fluorescent signal.
2. Pro-Q destaining buffer (25 mL): 20 % acetonitrile in sodium acetate (50 mM, pH 4).

2.6 TCPTP Phosphatase Activity Assay on the Microarray Slide

1. Blocking solution: 1 % BSA in TBS.
2. TCPTP activity buffer: 50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 0.01 % Brij 35, 1 mM DTT, pH 7.0.
3. TBS buffer.
4. TBST buffer: TBS buffer containing 0.05 % of Tween 20.

2.7 Solution-Based Microplate Assay

1. Malachite green assay kits (i-DNA Biotechnology Pte Ltd, Cat. No. POMG-25H).
2. Microplate shaker.
3. Tecan microplate reader (Tecan Group Ltd, Switzerland).
4. Greiner 384-well transparent plates (Practical Mediscience Pte Ltd).
5. Multichannel pipette (Practical Mediscience Pte Ltd).

2.8 Software

1. Array-Pro-Analyzer software (*Tecan* Trading *AG,* Switzerland).
2. GraphPad Prism v4.03 software (GraphPad, San Diego, USA).
3. Microsoft Excel.

2.9 General Apparatus

1. Adhesive film (ABgene, Cat. No. AB-0558).
2. Centrifuge (Eppendorf, 5415 R and 5810 R).
3. 15 and 50 mL centrifuge tubes.
4. Centrifuge filters (Microcon YM-3, cutoff 3 kDa).
5. Microscope glass slides: 75 mm × 25 mm × 1 mm.
6. Coverslips: 22 mm × 60 mm and 22 mm × 22 mm coverslips.
7. Marker pen.
8. Dessicator/dry storage box.
9. Ice box.
10. Humidity incubation chamber.

11. Powder-free gloves.
12. 1.5 mL reaction tubes and 14 mL sterile PP tube.
13. Slide-A-Lyzer dialysis cassettes 7K MWCO, 0.1–0.5 mL capacity (Thermo Scientific, Cat. No. 0066375).
14. Slide staining rack and slide staining jar.
15. Sonics Vibra-Cell sonicator (ITS Science & Medical Pte. Ltd.).
16. Microarray *Scanner* (Launch LS Reloaded, *Tecan* Trading *AG,* Switzerland).
17. UV/Visible spectrophotometer (Amersham Biosciences, Ultrospec 2100 Pro).
18. 96-well polypropylene stock plates: solid U-bottom and solid F-bottom (flat).

3 Methods

3.1 Expression of TCPTP

1. Transform competent *E. coli* BL21(DE3) with *TCPTP* construct and grow the single bacterial colony on LB-agar plates with kanamycin.
2. Inoculate a single bacterial colony from the agar plate into a 14 mL PP sterile tube containing 3–5 mL LB-kanamycin media. Grow the culture overnight at 37 °C in an orbital shaker with constant shaking at 200–250 rpm.
3. Dilute 100 times of the overnight culture into 200 mL fresh LB-kanamycin (50 μg/mL) media in a conical flask. Incubate the diluted LB culture at 37 °C with constant shaking at 200–230 rpm till OD_{600} reaches about 0.6–0.8 (it will take approximately 2–3 h).
4. Pipette a 10 mL culture of uninduced *E. coli* cells to be kept as a negative control for TCPTP expression.
5. The optimum absorbance (OD_{600}) for induction is 0.6–0.8. Check the absorbance periodically. If the cells are overgrown, repeat **steps 3** and **4**. Add IPTG (a final concentration of 0.1 mM) to induce TCPTP expression. Incubate the LB culture overnight (12–20 h) at 16 °C with constant shaking at 230 rpm.
6. Collect a 100 mL of induced LB culture, centrifuge, discard the supernatant, and store the cell pellets at −20 °C before use. Cell pellets can be stored at −20 °C for up to 1 month without any significant degradation of proteins. However, long-term storage (more than 3 months) of pellets may result in a decrease in protein activity.
7. Resuspend the induced bacterial cell pellets in 5–10 mL ice-cooled (4–8 °C) His-tag lysis buffer, and vortex if necessary.

3.2 Purification of TCPTP

1. Lyse the induced bacterial cells by sonicating them on ice at 30 % amplitude in a sonicator (5 s × 5 bursts, 10 s × 1 interval between each burst).
2. Centrifuge the bacterial lysate at 16,100 × *g* for 30 min at 4 °C. Transfer the supernatant to a fresh 1.5 mL tube on ice.
3. Pipette suitable amount of Ni-NTA resin beads into a chromatographic column (according to the expression levels, 100 mL of bacterial culture may need 150 μL of bead volume). Rinse the Ni-NTA resin with lysis buffer for 3–5 times (5–10 mL each time) before incubation with lysate.
4. Load the cell lysate and incubate it with the resin for about 45 min to 1 h with gentle shaking at 4 °C (*see* **Note 1**). The target protein TCPTP will bind to the Ni-NTA resin.
5. After incubation, let the lysate flow through the column slowly, and wash the column with His-tag wash buffer for 3–5 times (5–10 mL each time) (*see* **Note 2**).
6. Elute the target protein TCPTP with 0.3–1 mL of His-tag elution buffer for 2–4 times. When necessary, incubate the elution buffer with the resin for 2–5 min, and then collect all fractions of the eluent.
7. Measure the protein concentration by Bradford assay and combine the fractions that contain the highest amounts of TCPTP proteins (usually the first few fractions).
8. Run SDS gel electrophoresis and perform Western blotting to confirm the successful purification of TCPTP (Fig. 3).

3.3 Functionalization of Avidin onto the Surface of Microarray Slides

1. Carefully prepare piranha solution by slowly adding H_2O_2 to H_2SO_4 (*see* **Note 3**).
2. Place the slides in a tray and soak them in the piranha solution, and then occasionally shake the slides up and down.
3. Soak the slides for at least 4 h, and then take out the slides.
4. Carefully rinse the slides, first with H_2O and then with ethanol. Then dry the slides by flushing nitrogen gas or putting them in the fumehood for about 10 min till the slides are dry.
5. Prepare the APTES solution and pour it into a glass jar.
6. Put the glass slides into the glass jar with a stir bar, and then keep stirring the solution for 2 h.
7. After stirring, take out the slides and carefully wash them with 95 % ethanol for three times.
8. After washing, put the slides in a glass slide dish (with the lid) and then place it in a 150 °C oven for at least 2 h or overnight (*see* **Note 4**).
9. Take the slides out, and let them cool to room temperature.

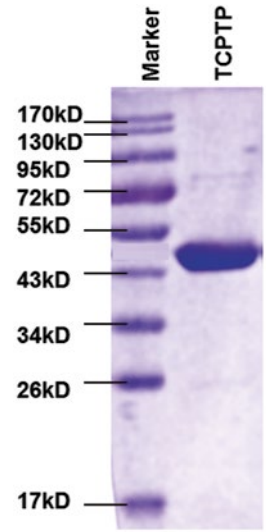

Fig. 3 Purification of TCPTP. This is a Coomassie blue-stained gel of the purified TCPTP protein

10. Wash the slides with 95 % ethanol three to five times (ethanol wash bottle) and dry the slides as described above.
11. Fill the slide tray with 400 mL of DMF and add 7.4 g of succinic anhydride. Put in a stir bar to stir the solution. After the solid is completely dissolved, add 18 mL of 1 M $Na_2B_4O_7$ (boric acid + NaOH, pH 9), and subsequently soak the slides into the solution (*see* **Note 5**). This step is for carboxylic acid activation.
12. Shake the slide tray once in a while to avoid small air bubbles adhering on the slides.
13. Meanwhile, start heating up 2 L of deionized water (use a stir bar) until it reaches about 95 °C.
14. Soak the slides in the hot water for 20 min with constant stirring (*see* **Note 6**).
15. Take out the slides and rinse them three to five times with ethanol using a wash bottle. Then briefly dry them with nitrogen gas or place the slides in the fumehood for about 10 min till the slides are dry.
16. Prepare the NHS activation solution and add 0.75 mL of the solution onto each slide.
17. Place the large coverslips (22 mm × 60 mm) on the slides, close the lid of the glass slide dish, and incubate for at least 3 h.
18. Transfer the slides to a metallic tray.
19. Wash the slides three to five times with 95 % ethanol using a wash bottle. This step takes about 3–5 min.

20. Prepare 1 mg/mL of avidin solution in 10 mM $NaHCO_3$.
21. Apply about 49 μL of the avidin solution onto each slide, and then incubate for 30 min under a coverslip (22 mm × 60 mm).
22. After the avidin immobilization reaction, quench the unreacted NHS groups with the quenching solution for 30 min and rinse the slides thoroughly with water.
23. Dry the slides using a stream of nitrogen gas, and store them at 4 °C before use.

3.4 Phosphopeptide Spotting and Quality Control

1. Prepare 16 μL of each biotinylated peptide solution in PBS/DMSO (1:1) to reach a final concentration of approximately 1 mM. This concentration would completely saturate the avidin group on the slides and it ensures that an adequate amount of peptides are immobilized (*see* **Note 7**).
2. Prepare the biotinylated peptide library in a 384-well polypropylene microarray plate for spotting, which is compatible with the microarray spotter.
3. Seal the spotting plate with adhesive film and store it at −20 °C. Thaw the plate when needed.
4. Before spotting, briefly wash the avidin-coated slides with deionized H_2O, and then quickly dry them with nitrogen gas or put them in the fumehood for about 10 min till the slides are dry.
5. Spot the biotinylated peptide library to the avidin-coated slides using the arrayer. After the spotting completes, the slides are allowed to stay inside the spotter for another 2–4 h. This will provide sufficient time for biotin/avidin interaction.
6. Put the slides into a slide dish containing 20 mL of deionized water and shake for at least 10 min to remove unbound peptides. Rinse the slides with distilled water. After the slides are briefly dried, store them at 4 °C before use.
7. To test the spotted slides and ensure the good quality of any given batch (i.e., the immobilization of phosphopeptides onto the surface of the avidin-coated slides is consistent between different batches), perform the Pro-Q™ assay as follows.
8. Dry the spotted slides by putting them in the fumehood for 10 min or using a stream of nitrogen gas.
9. Pipette 1 mL of Pro-Q™ Diamond dye and apply it onto the surface of the spotted slides for 1 h in a humidified incubation chamber under the coverslips (22 mm × 60 mm) at room temperature.
10. Wash the spotted slides with the Pro-Q™ destaining solution for 0.5–1 h. After washing, scan the slides with the microarray scanner. A typical image of Pro-Q™ stained slide is shown in Fig. 4.

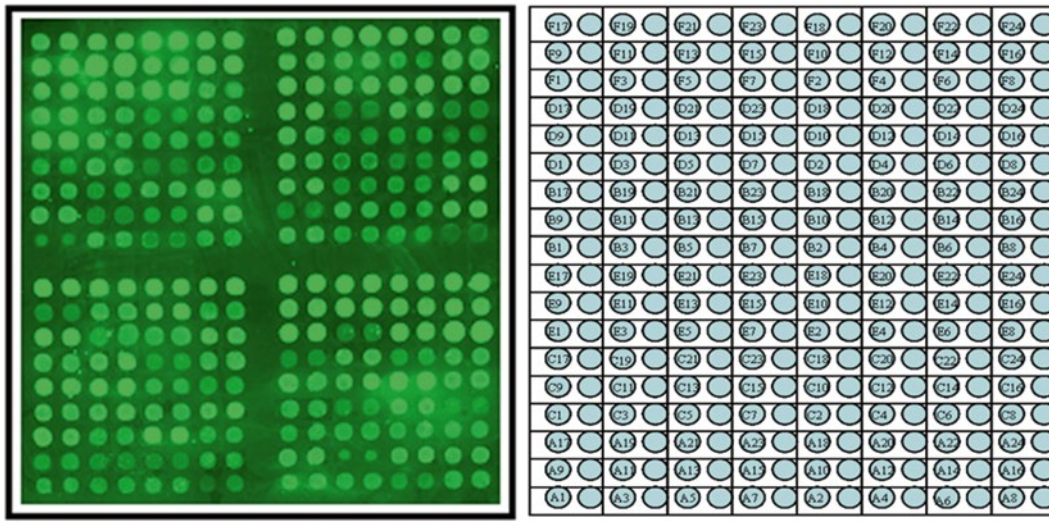

Fig. 4 Pro-Q staining image (*left*) and microarray spotting format (*right*). Pro-Q staining is used to confirm the good quality of spotted slides. The slide comprises a 144-phosphopeptide library printed in duplicate (reproduced in part from ref. [4] with permission from John Wiley & Sons Inc.)

3.5 Screening of TCPTP Substrates Spotted on the Microarray Slide

1. Bring the spotted slides to room temperature. Rinse them with distilled H_2O and shake for about 10 min to remove the unbound peptides.
2. Apply the blocking solution to the spotted slide for 1 h.
3. Prepare the TCPTP activity buffer.
4. As negative controls, use denatured TCPTP (heating at 95 °C for 10 min followed by incubation on ice) or activity buffer alone (*see* **Note 8**).
5. Perform the single-time-point microarray experiments to delineate the substrate specificity of TCPTP. Typically, the incubation time is 0.5 h and the volume of TCPTP applied to the slide is 75 μL. The enzyme can be applied directly onto the grid which is marked using a permanent marker pen to form rectangular frames. No coverslip is used.
6. After incubation, wash the slides with deionized H_2O to remove the TCPTP solution.
7. Gently rinse slides with deionized H_2O, and then quickly dry the slides with nitrogen gas or in the fumehood.
8. Perform the Pro-Q assay, which has been described in Subheading 3.4, **steps 7** and **10**.
9. After the Pro-Q assay, wash the slides with deionized H_2O to remove the Pro-Q reagent. When necessary, wash with TBST buffer to reduce the background so as to increase the signal-to-noise ratio (*see* **Note 9**).

10. Dry the slides with nitrogen gas or in the fumehood, and then scan them using microarray scanner to obtain the microarray image (Fig. 2).
11. Analyze the microarray images and perform quantitative data analysis. Confirm the well-known TCPTP peptide substrate (D14: TDKEYpYTVKDD) [19]. Identify unknown substrates (e.g., A06, D02) of TCPTP. In addition, the Top-10 peptide substrates identified from the single-time-point experiments for TCPTP are listed in Table 1.
12. To guide the design of potent inhibitors for PTPs in the future, perform further data analysis of the amino acid preference of the phosphopeptides at each position (namely from −5 to +5 positions, or from P−5 to P+5, counting from N- to C-terminus, with the location of phosphotyrosine (pY) defined as P) with the Top-10 peptides for TCPTP and the results are graphically presented in Fig. 5.
13. To better understand the kinetic information of TCPTP, time-course TCPTP activity assays are carried out for the Top-5 peptide substrates identified from the single-time-point microarray experiments. Different subgrids of peptide array were incubated with TCPTP solution in a humidified chamber for different time ranging from 5 min to 2 h (*see* **Note 10**).

Table 1
Top-10 peptide substrates identified from the single-time-point microarray experiments for TCPTP

Ranking	TCPTP
1	DSGGF*pY*TTSRT (**A10:SRC1**)
2	EEEPV*pY*EAEPE (**F18:HS1**)
3	REGLN*pY*MVLAT (D02:ROS1)
4	DEKVD*pY*VQVDK (**A06:GAB2**)
5	DEELH*pY*ASLNF (**A04:CD33**)
6	VYESP*pY*SDPEE (**D22:ZAP70**)
7	TNDIT*pY*ADLNL (**D16:SIRP**)
8	SDDVR*pY*VNAFK (**D04:VEGF**)
9	VSSTH*pY*YLLPE (**F23:CDC42**)
10	MTGDT*pY*TAHAG (**C05:ABL**)

pY represents phosphotyrosine. The note underneath the peptide sequence indicates the peptide ID, and the original protein source (reproduced in part from ref. [4] with permission from John Wiley & Sons Inc.)

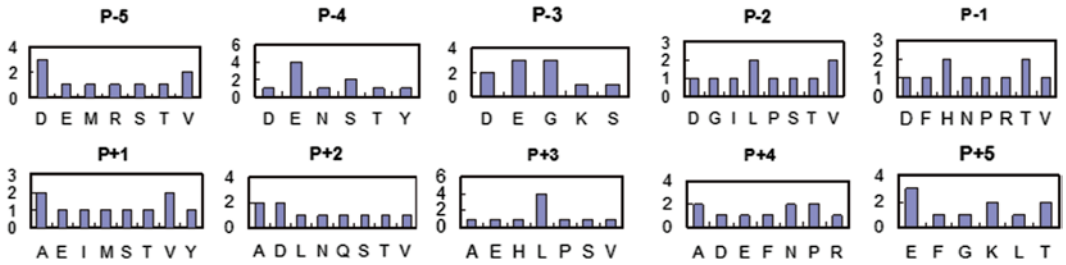

Fig. 5 Summary of amino acid preference of the phosphopeptides from their −5 to +5 positions (e.g., P + 5 is 5 amino acid positions toward the C-terminus with phosphotyrosine located at the P position). The data are extrapolated from the Top-10 peptide substrates (*see* Table 1) identified from the single-time-point experiments using TCPTP

14. After the TCPTP incubation, stain the slides by repeating the same **steps 6–10**. The time-dependent microarray images are shown in Fig. 6a.
15. Analyze the array images and perform quantitative data analysis. Calculate the corresponding k_{obs} value using the GraphPad software (Fig. 6b and Table 2). The larger the k_{obs} value is, the faster TCPTP will dephosphorylate the corresponding phosphopeptide.

3.6 Validation of the Identified Hits Using the Solution-Based Microplate Assay

1. Transfer 20 μL of TCPTP activity buffer containing 20 μM of the peptides into each well of a transparent 384-well microplate.
2. Pipette the TCPTP solution (20 μL) into each well at different time points (0, 5, 15, 30, 60, and 120 min). The kinetic data for dephosphorylation of each phosphopeptide at each time point are determined in duplicates.
3. Stop the dephosphorylation reaction by addition of malachite green solution which contains sulfuric acid to denature the TCPTP. If the reaction solution contains free phosphate groups, which are generated through dephosphorylation of the phosphopeptide substrates by TCPTP, the reaction solution will then become green in color.
4. When the green color of the reaction solution become stable (around 10 min), measure the absorbance of the malachite green-phosphate complex at 650 nm using a microplate reader.
5. Perform background subtraction ($t = 0$ min) and data normalization.
6. Process microplate data with the software GraphPad and fit the data with the following equation to plot the kinetic curves and derive the kinetic parameter k_{obs} (Fig. 6c and Table 2):

$$\mathrm{Abs}_{obs} = \mathrm{Abs}_{max} \times \left(1 - \exp\left(-k_{obs} \times t\right)\right).$$

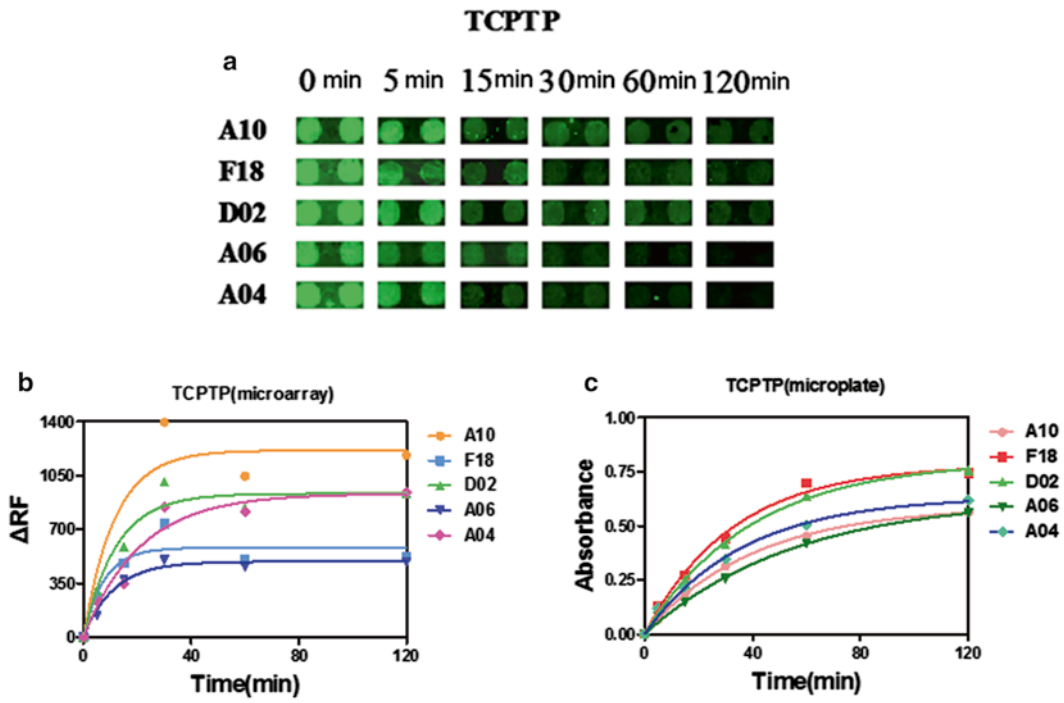

Fig. 6 Time-dependent, semi-quantitative dephosphorylation experiments of selected top five peptides with TCPTP. (**a**) Microarray images of selected spots from the time-dependent kinetic experiment of top five peptides (*see* Table 1) against TCPTP; (**b**) Plots of extrapolated microarray data and k_{obs} fitted curves; (**c**) Microplate-based time-dependent experiments (k_{obs} fitted curves) of the same five peptides against TCPTP (reproduced in part from ref. [4] with permission from John Wiley & Sons Inc.)

Table 2
Summary of kinetic constants (k_{obs}) determined from the microarray and microplate experiments for Top-5 peptide substrates (*see* Table 1) against TCPTP (reproduced in part from ref. [4] with permission from John Wiley & Sons Inc.)

TCPTP	k_{obs} (array, min^{-1})	k_{obs} (plate, min^{-1})
A10	0.086	0.025
F18	0.132	0.03
D02	0.082	0.025
A06	0.089	0.018
A04	0.047	0.027

where Abs_{obs} represents the absorbance reading at time x; Abs_{max} represents the fitted constant which corresponds to the maximum absorbance obtained when the enzymatic reaction is complete; t is the time.

Good agreement in the k_{obs} values is obtained for most of the phosphopeptide/PTP pairs between the time-dependent microarray-based experiments and the standard microplate assays (Table 2).

In summary, a phosphopeptide microarray-based approach has been described for high-throughput screening of substrate specificity of TCPTP. By this approach, we have successfully obtained substrate fingerprints for TCPTP, amino acid preference of TCPTP at each position, as well as the kinetic constants of peptides substrates for TCPTP. The high-throughput substrate fingerprint strategy can also be applied for studying other PTPs or Ser/Thr phosphatases.

4 Notes

1. To avoid the decrease in the enzymatic activity of TCPTP, carry out this step of incubation at 4 °C.
2. The columns are designed for low-pressure applications (less than 1 atm or 14 p.s.i.). When a pump is used for the liquid flow, any disruption in the flow must be avoided since this disruption may cause a rapid increase in back pressure. If this increase does happen, immediately switch off the pump and check the gel bed for liquid leakage.
3. When preparing the piranha solution, pour H_2SO_4 in the beaker first and then add H_2O_2. Make sure the temperature of the solution does not increase too much since it can get extremely hot. Be careful when handling the piranha solution since this hot acid solution is extremely corrosive.
4. To prepare the slides with high-quality surfaces, avoid sticking the slides to each other when putting the slide rack in the 150 °C oven.
5. Always immerse the slides in the reaction solution. Otherwise, the slide will be unevenly cleaned, and this will lead to uneven surfaces for the subsequent preparation of avidin slides, which will greatly compromise the quality of microarray.
6. Similarly, keep the slides immersed in water at all times. Otherwise, the surfaces of the slides may not be uniformly cleaned, which would greatly compromise data quality of the subsequent microarray experiments.
7. To prepare the spotted microarray slides with good quality, prepare the peptide solution at a high concentration (i.e., not

less than 1 mM) for spotting. Otherwise, the amount of peptides may not be sufficient to saturate the slide surface during immobilization.

8. There should be no (or negligible) signal difference between slides treated with negative controls (denatured TCPTP or activity buffer solution alone) and untreated slides. A decrease in fluorescent signals (stained using the Pro-Q dye) will be observed if the TCPTP is active and the phosphopeptides are the substrates of TCPTP so that the phosphate groups are removed from the peptides.
9. The frequency and duration of slide washing could be optimized as required. We typically use one to three times of 5-min washes which would produce very good results.
10. When the coverslips are put on the slides, care must be taken that no air bubbles are trapped between the coverslips and the slides. Otherwise, no dephosphorylation reaction would occur and this would generate false negatives. Besides, to acquire good and consistent fingerprint results with low backgrounds, carefully apply the TCPTP solution uniformly across the surface of the slides.

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